

# Immunosuppressive effects of tautomycetin *in vivo* and *in vitro* via T cell-specific apoptosis induction

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**Tautomycetin (TMC) was identified as an immunosuppressor of activated T cells. Inhibition of T cell proliferation with TMC was observed at concentrations 100-fold lower than those needed to achieve maximal inhibition with cyclosporin A (CsA). TMC specifically blocked tyrosine phosphorylation of intracellular signal mediators downstream of Src tyrosine kinases in a T cell-specific manner, leading to apoptosis due to cleavage of Bcl-2, caspase-9, caspase-3, and poly(ADP-ribose) polymerase, but not caspase-1. In TMC-treated rats that received a heterotopic cardiac allograft, the graft survived more than 160 days, comparable to graft survival in allografted rats treated with CsA. Thus, TMC, whose mechanism of action is different from that of CsA or FK506, can be used as a potent T cell-specific immunosuppressor.**

Organ transplantation to replace diseased organs has become the standard treatment in terminal organ failure, such as in renal, hepatic, or cardiac diseases (1). In most cases, induction of T cell-mediated immune responses to the highly polymorphic MHC molecules on nucleated cells in the grafted organ is the major barrier to successful transplantation (2). Improvement in graft survival is accomplished by precise HLA typing capability, greater surgical experience and skill, and the potential of cloned animals as organ donors. But genetic differences at loci other than MHC still trigger rejection. Therefore, continuous efforts to discover effective and specific immunosuppressive agents have been intense.

The systematic study of products from bacteria and fungi has led to the development of immunosuppressive drugs such as cyclosporin A (CsA), FK506 (tacrolimus), and rapamycin (3). CsA and FK506 block T cell activation by preventing the induction of IL-2 gene expression, whereas rapamycin blocks the signaling pathway triggered by IL-2 receptor (4). They exert their pharmacological effects by binding to the immunophilins, and the immunophilin and drug complex binds and inhibits the Ser/Thr phosphatase calcineurin, which is activated when intracellular calcium ion level rises on T cell activation (5, 6). Rapamycin has a different mode of action from either CsA or FK506. Like FK506, rapamycin binds to the FK506-binding protein family of immunophilins. However, the complex of rapamycin/immunophilin has no effect on calcineurin activity but instead blocks the signaling pathway triggered by the IL-2 receptor. These drugs are effective immunosuppressive agents, but they are not free of problems. Because calcineurins are found in many cells, these drugs are expected to be deleterious in many other tissues, such as kidney and liver (7). Therefore, it is important to develop new immunosuppressors with minimal toxicity that target molecules specifically involved in immune responses. It would also be helpful to have more than two different immunosuppressive drugs with different pharmacological effects.

In this study, we identified tautomycetin (TMC) as an activated T cell-specific immunosuppressor. Being different from CsA and FK506 in its mechanism of action, TMC inhibited the induction of tyrosine phosphorylation of T cell-specific signaling mediators in T cell receptor (TcR) proximal signal transduction pathway, leading to induction of apoptosis. In *in vivo* studies, TMC was at least as effective as CsA in the heterotopic cardiac transplant animal model.

## Materials and Methods

**Cell Culture and Reagents.** Human peripheral blood lymphocytes from healthy volunteers were isolated by standard Ficoll-Hypaque gradient centrifugation. The cell lines HeLa, Jurkat, and CD8- $\zeta$  Jurkat transfectant were cultured in standard medium containing FBS. Jurkat transfectants were regularly treated with medium containing 2.2 mg/ml of Geneticin (GIBCO/BRL). OKT3 and -8 mAb were purified from supernatants secreted from OKT3 and -8 hybridomas (American Type Culture Collection) by Gamma Bind plus Sepharose from Pharmacia Biotech. The following antibodies were used: anti-p-Tyr (4G10; Upstate Biotechnology, Lake Placid, NY), anti-p-Tyr agarose conjugated beads (Upstate Biotechnology), anti-ICE (Upstate Biotechnology), anti-Fyn (Upstate Biotechnology), anti-phospho-ERK-1/-2 (New England Biolabs), anti-Cpp32 (Transduction Laboratories, Lexington, KY), anti-Caspase-9 (PharMingen), anti-CD3 $\zeta$  (ID4; PharMingen), anti-CD28 (PharMingen), and anti-CD69 (FN50; PharMingen). Antibodies of anti-Bcl-2 (100), anti-Cbl (C-15), anti-LAT (Q-20), anti-poly(ADP-ribose) polymerase (PARP) (H-250), anti-PLC $\gamma$ -1 (530), anti-SLP-76 (K-20), anti-Vav (C-14), and anti-ZAP70 (LR) were purchased from Santa Cruz Biotechnology. Phorbol 12-myristate 13-acetate and Concanavalin A (ConA) were obtained from Sigma. CsA was generously provided by Chong Kun Dang (Seoul, Korea), and tautomycin was purchased from WAKO Biochemicals (Osaka).

**Separation and Purification of TMC.** *Streptomyces* sp. CK-4412, isolated from forest soil on Cheju Island, Korea, was cultured at 28°C for 65 h in Erlenmeyer flasks containing a medium composed of soluble starch, 3%; soybean flour, 1.5%; corn steep liquor, 1.5%; polypeptone, 0.2%; Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.1%; CaCO<sub>3</sub>, 0.5%; and allophosite, 1%. After centrifugation, the supernatant (6.9 liters) was extracted with EtOAc, and the extracts were evaporated *in vacuo*. The extracts were then applied to LH-20 and silica gel column developed with the solvent, CHCl<sub>3</sub>:*n*-hexane:acetone:AcOH (10:5:3:0.1). Active fraction called CKD-722 was collected and concentrated *in vacuo* to give a yellowish oily material, and further purification and identification were performed with HPLC by using an octadecyl silane (ODS) column, NMR, and electrospray ionization-MS analysis.

**Murine Mixed Lymphocyte Reaction (MLR) Assays and IL-2 ELISA.** For murine MLR assays, the immunosuppressive activity of active fractions and TMC was assessed with mixed lymphocyte reactions, as previously described (8). For IL-2 ELISA, 1  $\times$  10<sup>5</sup> human primary T cells were activated by using 1  $\mu$ g/ml of OKT3 and 0.5

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Abbreviations: TMC, tautomycetin; CsA, cyclosporin A; PI, propidium iodide; MLR, mixed lymphocyte reaction; BcR, B cell receptor; TcR, T cell receptor; PARP, poly(ADP-ribose) polymerase.

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Corrected value of absorbance = absorbance at 570 nm – absorbance at 450 nm.

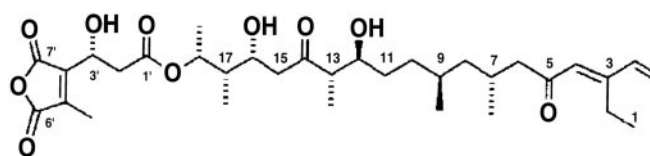
**Heterotopic Cardiac Allograft.** The microsurgical technique of heart transplantation in rat was done by double anastomosis. With ether anesthesia, the abdomen of the Lewis rat was opened and bled by cutting the abdominal aorta and its chest opened. After perfusion of heart through the ascending aorta with cold saline solution, the heart was removed from the mediastinum after the division of the trachea and placed in a beaker containing cold saline. The abdomen of the recipient Wistar rat was opened by midline incision. The Lewis heart was placed within the abdominal cavity, and end-to-side anastomosis was done by sewing the pulmonary artery and aorta to the inferior vena cava and abdominal aorta of the Wistar, respectively. Soon after perfusion, regular beating of the heart followed the ventricular fibrillation. The intestine was returned to the abdomen, and the abdominal wall was closed.

**Apoptosis Assays.** Cell death was confirmed by detecting DNA fragmentation. Briefly,  $5 \times 10^6$  cells were lysed in  $\times 2$  lysis buffer (200 mM Hepes, pH 7.5/2% Triton X-100/400 mM NaCl/20 mM EDTA) and incubated with RNase at 37°C for 1 h. DNA was extracted with phenol and precipitated with 5 M ammonium acetate and 2.5 volume of 100% ethanol and then analyzed by electrophoresis on 2% agarose gel. Also, for cell viability analysis,  $1 \times 10^6$  cells were determined by staining with propidium iodide (PI) and analyzed on a FACScan (Becton Dickinson).

**Transient Transfection.** Jurkat TAG cell lines were transiently transfected with 5  $\mu$ g of plasmid DNA by using Superfect transfection reagent (Qiagen, Hilden, Germany). Cells ( $3 \times 10^6$ ) were washed once with PBS and plated to a 60-mm dish. Plasmid DNA was incubated for 10 min at room temperature in serum-free medium containing Superfect. After the DNA-Superfect complexes were added dropwise to the cells in a 60-mm dish, the cells were incubated in fresh medium for 36 h.

## Results

**Identification of TMC as an Immunosuppressor.** We isolated a large number of actinomycete strains from forest soil on the volcanic

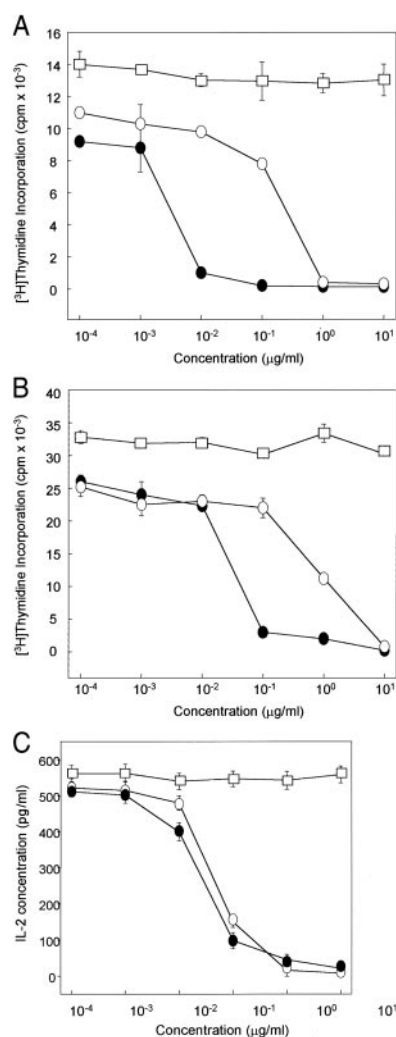


**Fig. 1.** Structure of TMC.

Cheju Island in Korea. Appropriate dilutions of culture broth after centrifugation were included in mouse MLR to examine their inhibitory effects on T cell proliferation. The culture broth of *Streptomyces* sp. CK-4412 showing the strongest inhibition of T cell proliferation in MLR was prepared on a large scale and then applied to an LH-20 and silica gel chromatography column. The active fraction, called CKD-722, was collected and further purified by HPLC by using an ODS column. CKD-722 prepared in MeOH showed characteristic UV absorption at 268 nm, and the main absorption band in IR spectrum appeared at 1,825, 1,760, 1,730, and 1,700  $\text{cm}^{-1}$ . From the analysis of electrospray ionization mass spectrometry ( $m/z$  661,  $\text{M} + \text{CH}_3\text{OH} + \text{Na}$ ) and  $^1\text{H}$  and  $^{13}\text{C}$  NMR, CKD-722 was identified as TMC (Fig. 1). TMC was originally found as a new antifungal antibiotic produced by *Streptomyces griseochromogenes* (8).

**Inhibition of T Cell Proliferation and Activation by TMC.** The inhibitory effect of TMC on T cell proliferation was confirmed in the MLR between splenocytes of BALB/c and mitomycin C-treated splenocytes of C57BL/6 in the presence of serial dilutions of TMC or CsA, the most frequently used immunosuppressor. The level of inhibition of MLR by TMC was 100-fold higher than that by CsA, and the  $IC_{50}$  of TMC and CsA were 7.8 and 417 nM, respectively (Fig. 2A). Similar levels of proliferation inhibition were observed in mouse splenocytes stimulated with the mitogen Con A (Fig. 2B). Also, similar kinetics of inhibition by TMC or CsA was observed in rat splenocytes. Induction of IL-2 gene expression, CD69, and IL-2R $\alpha$  chain surface expression has been well documented as valuable markers for TcR-distal activation events (9). The potency of TMC in the IL-2 secretion assay in human primary T cells is similar to that of CsA in contrast to its more potent inhibitory activity than that of CsA in murine MLR and murine spleen cell proliferation in response to Con A. This result demonstrated that TMC possesses immunosuppressive activity to inhibit the proliferation of T cells by inhibition of IL-2 secretion (Fig. 2C). In contrast to cells pretreated with CsA, the surface expression of CD69 or IL-2R $\alpha$  chain was not induced in Jurkat T cells pretreated with TMC after TcR stimulation (data not shown). These results indicated that TMC has the capacity to inhibit the intracellular signaling pathway leading to T cell activation and proliferation, and its mechanism of action might be different from that of CsA.

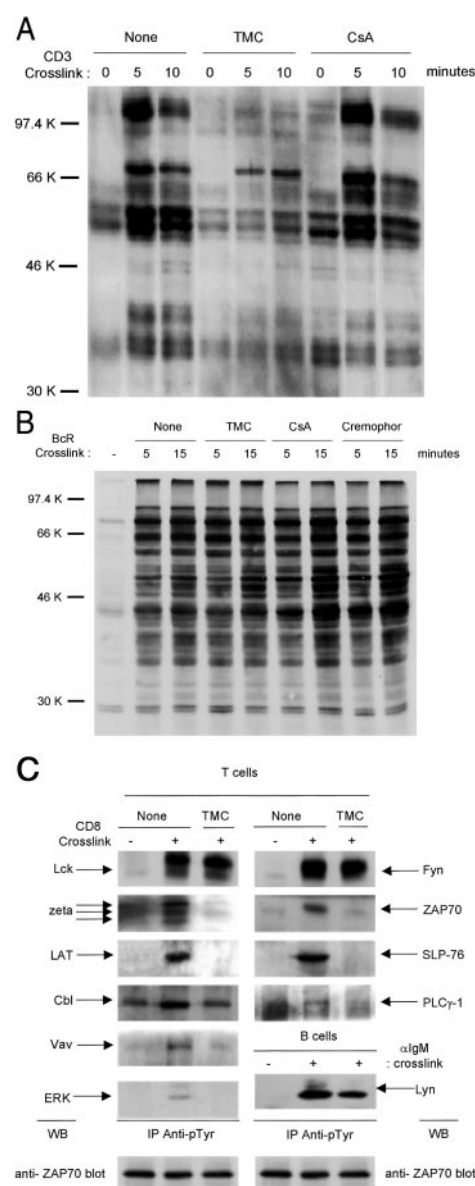
**TMC Inhibits T Cell-Specific Tyrosine Phosphorylation Induction in TcR-Proximal Signaling.** The membrane-proximal signaling events initiated by the TcR complex are activation and recruitment of protein tyrosine kinases and subsequent phosphorylation of cellular proteins (10, 11). To identify the signaling event targeted by TMC in the TcR-mediated signaling pathway, inhibition of tyrosine phosphorylation of various intracellular substrates by TMC was examined in primary human T cells. TMC blocked the phosphorylation of tyrosine residues on several specific cellular proteins in T cells stimulated by immobilized OKT3 mAb (Fig. 3A). The inhibition of tyrosine phosphorylation by TMC is dose- and time-dependent (data not shown), and induction of tyrosine phosphorylation was not influenced by CsA as expected. This result raises the possibility that TMC may block tyrosine phosphorylation of intracellular signal mediators like genistein or herbimycin (12). To rule out this possibility, induction of tyrosine phosphorylation of intracellular proteins was examined in the human primary B cells after



**Fig. 2.** Inhibition of proliferation and activation of T cells by TMC. (A) MLR was set up by culturing splenocytes of BALB/c mouse with mitomycin C-treated allogenic spleen cells of mouse C57BL/6 in the presence of TMC (●), CsA (○), or cremophor-EL only (□). (B) Spleen cells from BALB/c were stimulated with Con A (Sigma) for 48 h in the presence of TMC (●) (CsA (○) or cremophor-EL only (□), and then [<sup>3</sup>H] thymidine incorporation was measured. (C) Human primary T cells ( $1.0 \times 10^5$ ) were pretreated with TMC (1 μg/ml, ●), CsA (1 μg/ml, ○), or cremophor-EL only (□), and then stimulated with OKT3 mAb (10 μg/ml) and anti-CD28 mAb (0.5 μg/ml) at 37°C for 24 h.

B cell receptor (BcR) stimulation by anti-BcR mAb (13). As shown in Fig. 3B, TMC, CsA, or solubilizing reagent cremophore-EL did not affect the BcR-induced tyrosine phosphorylation of intracellular signaling molecules. As expected, genistein inhibited the induction of tyrosine phosphorylation in both T and B cells on receptor stimulation (data not shown). Specificity of TMC on activated T cells was further confirmed in Fig. 4B, where activated or resting human primary T and B cells were used. Therefore, TMC specifically inhibits the tyrosine phosphorylation of T cell activation-signaling pathway.

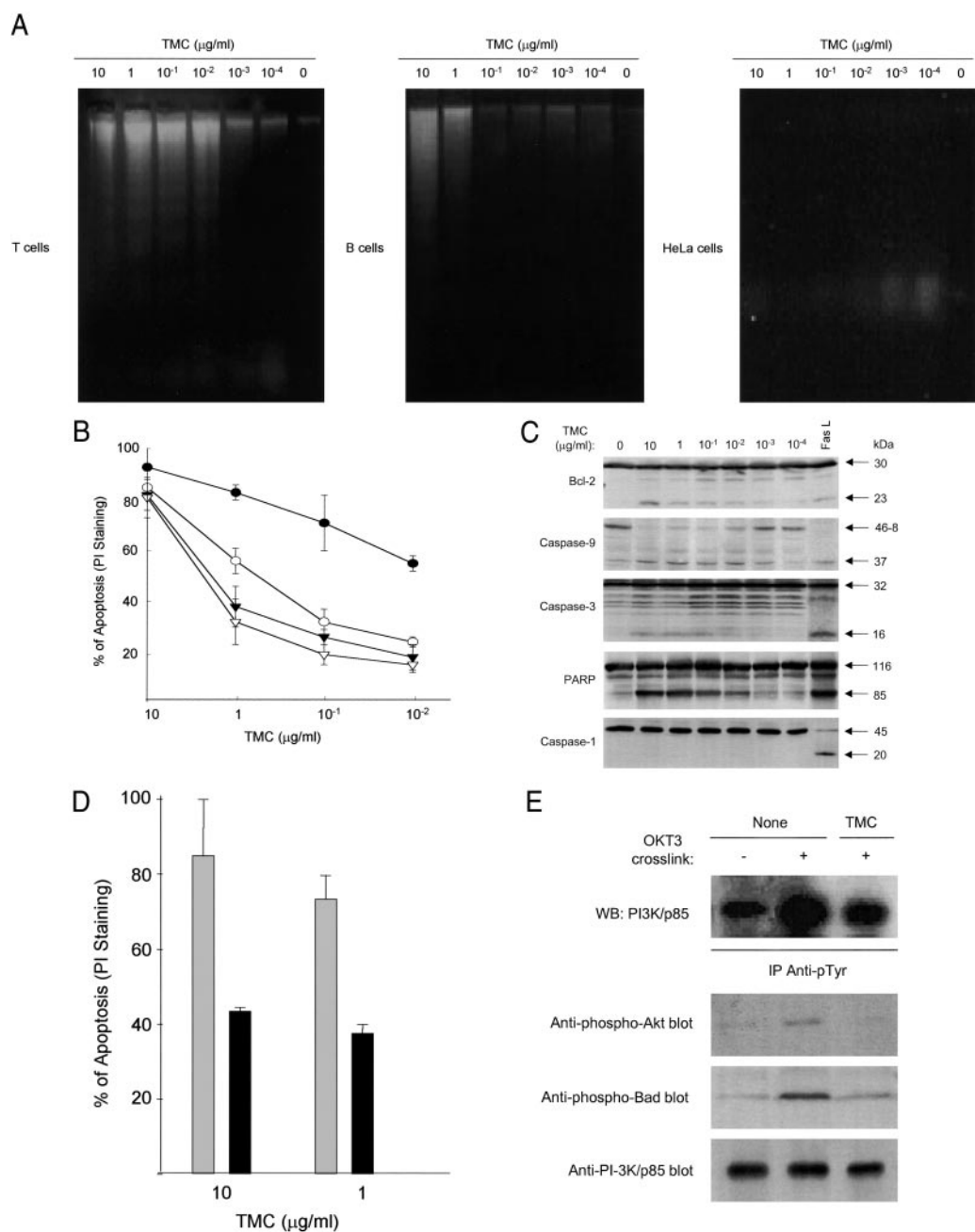
To identify the target molecule affected by TMC, we examined tyrosine phosphorylation induction of several key molecules participating in TcR-proximal or -distal signaling events in the presence of TMC by using the CD8-ζ Jurkat transfectant. In this transfectant, induction of tyrosine phosphorylation of intracellular proteins was observed on stimulation of CD8-ζ chimera by OKT8 mAb, whose pattern was qualitatively and quantitatively similar to that induced



**Fig. 3.** T cell-specific inhibition of tyrosine phosphorylation on intercellular signal mediators by TMC. (A) Human primary T cells were pretreated with TMC (1 μg/ml) or CsA (1 μg/ml) for 5 h. Then cells were stimulated with immobilized OKT3 mAb (10 μg/ml) and rabbit anti-mouse IgG (10 μg/ml) for the indicated times. (B) Human primary B cells were pretreated with TMC (1 μg/ml), CsA (1 μg/ml), or solubilizing reagent (cremophore) for 5 h and then stimulated with goat anti-human IgM F (ab')<sub>2</sub> (10 μg/ml) for the indicated times. (C) CD8-ζ Jurkat transfectants or primary human B cells were pretreated for 5 h with or without TMC (1 μg/ml) and then stimulated with OKT8 mAb (10 μg/ml) and rabbit anti-mouse IgG (10 μg/ml) or goat anti-human IgM F (ab')<sub>2</sub> (10 μg/ml) for 5 min, respectively. Immunoprecipitates or total lysates were resolved by SDS/PAGE and immunoblotted with the indicated antibodies. Treatment of cells with these drugs for 5 h did not induce cell death. An equal amount of protein was loaded in each well, which was confirmed by Ponceau S staining and immunoblotting with the anti-ZAP-70 mAb.

by TcR crosslinking (14, 15). In the presence of TMC, the intracellular domain of ζ chain, ZAP-70 tyrosine kinase, immune-specific adaptors such as LAT and SLP76, ubiquitous adapter c-Cbl, PLC-γ, Vav, and MAP kinase ERK were not tyrosine phosphorylated, which have been characterized to be downstream of Lck and Fyn in TcR signaling cascade (Fig. 3C) (16). Surprisingly, tyrosine

**Fig. 4.** T cell-specific induction of apoptosis by TMC. (A) The primary T and B lymphocytes and HeLa cells were incubated for 10 h with the medium alone or various concentrations of TMC. Cells were lysed, and DNA fragmentation was analyzed by electrophoresis. (B) The primary human T lymphocytes were preincubated without (○) or with (●) immobilized OKT3 mAb (10  $\mu$ g/ml), and B lymphocytes were preincubated without (▽) or with (▼) goat anti-human IgM F(ab')<sub>2</sub> for 3 h, and then treated with TMC (1  $\mu$ g/ml) for 5 h. Cell viability was determined by staining with PI and measurement by flow cytometry. (C) Jurkat T cells were incubated with various concentrations of TMC for 5 h. Cell lysates were subjected to SDS/PAGE under reducing condition (anti-PARP blot, anti-caspase-3 blot, anti-caspase-9, and anti-Bcl-2 blot), and native PAGE under nonreducing conditions (anti-caspase-1 blot), and then immunoblotted with the indicated antibodies. As positive controls for the cleavage of these molecules, cells were stimulated with NIH 3T3 transfectant stably expressing human FasL. (D) Jurkat T cells (3  $\times$  10<sup>6</sup>) stably expressing high level of SV40T antigen were transfected by using Superfect transfection reagent with null vector (gray) or bcl-2 expression vector (black). Cells were treated for 5 h with TMC (1 or 10  $\mu$ g/ml) after transfection and then analyzed by PI staining assay. (E) The primary human T lymphocytes purified from peripheral blood using Ficoll were pretreated with TMC (1  $\mu$ g/ml) for 5 h. Cells were then stimulated with immobilized OKT3 mAb (10  $\mu$ g/ml) for 5 min. Lysates were immunoprecipitated with anti-p-Tyr-conjugated agarose beads, and the precipitates were immunoblotted with anti-PI-3K/p85 mAb. For anti-phospho-Akt, anti-phospho-Bad, and anti-PI-3k/p85 blots, the lysates were immunoblotted with the indicated antibodies. An equal amount of protein was added in each lane, which was confirmed by Ponceau S staining and immunoblotted with the anti-PI-3K/p85 mAb.



phosphorylation of Lck and Fyn and their kinase activity was not influenced, suggesting that TMC targets a signal mediator between ZAP-70 and Lck or Fyn tyrosine kinase in TcR-mediated signal transduction cascade. A similar pattern of tyrosine phosphorylation inhibition was observed with primary human T cells stimulated by OKT3, and anti-CD28 mAb in the presence of TMC (Fig. 6, which is published as supporting information on the PNAS web site, www.pnas.org). However, Lyn tyrosine kinase specifically induced on BcR stimulation was not influenced by TMC.

**T Cell-Specific Induction of Apoptosis by TMC.** Triggering programmed cell death in activated T lymphocytes is one of the most important regulatory mechanisms of the immune response (17). TMC induced DNA fragmentation of primary T cells using concentrations as low as 1.7 nM. Primary B cells showed minimal cell

death at high concentrations of TMC, and apoptosis was not observed in HeLa cells in the presence of TMC (Fig. 4A). Induction of apoptosis was quantitatively confirmed by PI and annexin V staining, and CD4<sup>+</sup> and CD8<sup>+</sup> T cells did not show any difference in the level of apoptosis induction by TMC (Fig. 7, which is published as supporting information on the PNAS web site). The purified activated human T cells showed much higher sensitivity to TMC than resting T cells or activated or resting B cells (Fig. 4B). This result is consistent with the mechanism of action of TMC, which inhibits tyrosine phosphorylation induction of T cell-specific intracellular signal mediators.

Two independent apoptotic-signaling pathways triggered by the ligation of death receptors or various forms of cellular stress have been proposed, and these two pathways converge on the activation of downstream caspase-3, -6, -7, -9 (18). To investigate the molec-

**Table 1. *In vivo* immunosuppressive effect of tautomycetin on graft survival after heterocardiac transplantation in rat**

Drug	Concentration, mg/kg	Injection	Survival days	No. of rats
Cremophor-EL only	5	i.p.	9	4
	5	i.p.	10	3
CsA/cremophor-EL	5	i.p.	>100	2
Tautomycin/PBS	0.05	i.p.	10	3
TMC/PBS	0.05	i.p.	>160	12
TMC/ME	0.03	i.v.	>160	8

The Lewis rat heart was transplanted in the abdominal cavity of the Wistar rat by microsurgical anastomosis. After the heterotopic heart transplantation, the recipient was given CsA (5 mg/kg of rat weight optimally formulated in cremophor-EL solution) for 40 days, tautomycin (0.05 mg/kg of rat weight and solubilized in PBS), or TMC (0.03 or 0.05 mg/kg of rat weight and solubilized in PBS or in microemulsion form (ME) for 30 days. Graft survival was monitored by examining the heartbeat through the abdominal wall, and anatomical examination was done on 100th or 160th day after heterocardiac transplantation.

ular mechanism of apoptosis induction by TMC, cleavage of downstream effector caspases and their key substrates was examined (Fig. 4C). Bcl-2, which was known to bind to mitochondria and inhibit the release of cytochrome *c*, was cleaved as a consequence of TMC treatment in a dose-dependent fashion, and overexpression of Bcl-2 in Jurkat TAG cell line abrogated the apoptotic effect of TMC (Fig. 4D) (19). Similar kinetics of cleavage was observed in downstream caspase-9, caspase-3-like protease, and PARP. However, caspase-1-like protease (20), which was cleaved by natural Fas ligand stimulation, was not affected by TMC treatment. Z-VAD-fmk, which was known to inhibit the function of caspase-1 and -8, inhibited the TMC-induced apoptosis in a dose-dependent manner (Fig. 8, which is published as supporting information on the PNAS web site). This finding suggested involvement of caspase-8 in TMC-mediated apoptosis induction via mitochondria. These results indicated that T cell-specific induction of apoptosis by TMC is in part mediated by Bcl-2 cleavage, leading to the release of cytochrome *c*, which facilitates binding of Apaf- to caspase-9 in its presence and subsequent activation of caspase-8 and -3 in apoptosis induction signaling pathways.

In recent studies, the serine/threonine kinase Akt is emerging as a key molecule involved in regulating cell survival in a variety of models (21). To investigate the involvement of PI-3 kinase, Akt, and BAD in T cell-specific apoptosis induction by TMC, the induction of phosphorylation of PI-3 kinase, Akt, and BAD was examined in the presence of TMC by using the primary T cells stimulated by immobilized OKT3 mAb. As shown in Fig. 4E, TMC significantly inhibited the phosphorylation of Akt and BAD, suggesting that these three molecules might be responsible for the possible functional crosstalk between inhibition of T cell activation and induction of T cell-specific apoptosis by TMC.

**Immunosuppressive Effect of TMC *in Vivo*.** To assess the *in vivo* immunosuppressive effect of TMC, we measured allograft survival in rats treated with either TMC or CsA after heterotopic cardiac transplantation (22). The mean graft survival without administration of any immunosuppressor or solubilizing reagent was only 9.5 days, compared with more than 100 days for an isograft. The recipient was treated with CsA (5 mg/kg of rat weight) optimally formulated in cremophor-EL polyethoxyethylated castor oil solution for 40 days or with several different doses of TMC solubilized in PBS or in microemulsion form for 30 days after heterocardiac transplantation. As shown in Table 1, graft survival was prolonged for more than 100 days in the CsA-treated group. The grafted heart survived for more than 160 days in groups of rats treated with TMC using a dose as low as 0.03 mg/kg, and the level of graft survival was observed in a dose-dependent manner. On histological examination on the 160th day after transplantation, the cardiac muscle cells,

vascular architecture, and renal tubes were well preserved, and T cell infiltration was minimal in the recipient. Biochemical analysis of GOP, GTP, glucose, and creatine levels in blood after administration of TMC up to 5 mg/kg in rats for 30 days did not show any significant liver and kidney toxicity (see Table 2, which is published as supporting information on the PNAS web site). In contrast to TMC, tautomycin did not show any immunosuppressive effect in *in vivo* heart allograft transplantation. These results demonstrated that the *in vivo* immunosuppressive activity of TMC is as effective as CsA in an *in vivo* organ transplantation model, and the immunosuppressive effect of TMC can be improved even more if the pharmacological formulation of TMC is optimized.

## Discussion

A new *Streptomyces* strain was isolated from the soil of volcanic Cheju Island, which produces a novel immunosuppressive molecule. The active compound was purified through various chemical methods and identified as TMC, which was originally found as an antifungal antibiotic. A comparable level of inhibition of T lymphocyte proliferation induced by mouse MLR or the mitogen Con A was observed with a 100-fold lower concentration of TMC than CsA. Although the induction of IL-2 gene expression was inhibited by both TMC and CsA, TMC showed the inhibition of the surface expression of CD69 or IL-2R $\alpha$  chain. These findings suggested that TMC has the capacity to inhibit the intracellular signaling pathway leading to T cell activation and proliferation, and its mechanism of action is different from that of CsA.

Among the intracellular signaling events proximal to the TcR complex, TMC inhibited T lymphocyte activation by blocking the tyrosine phosphorylation induction of the T cell-specific signal mediator in a dose- and time-dependent manner on TcR stimulation. This inhibitory effect of TMC was not observed in primary B cells when BcR was engaged by anti-BcR mAb. Further analysis of the tyrosine phosphorylation state of individual key signaling molecules in the T cell activation pathway demonstrated that TMC inhibited T lymphocyte activation by blocking the tyrosine phosphorylation induction of T cell-specific signal mediators located between Lck or Fyn tyrosine kinase and ZAP-70 tyrosine kinase. The molecule downstream of these two kinases might be the target of TMC. A recent study supported the concept that TcR antagonism can occur through the generation of an inhibitory signal within the TcR complex, and the inhibitory effect can be exerted through the action of proteins that specifically bind partially phosphorylated  $\zeta$ . Binding proteins could be unknown inhibitory phosphatases brought into the vicinity of TcR or unknown positively acting kinases present in a limited amount. Alternatively, one of these binding proteins might be the target molecule of TMC.

Surprisingly, the T cell-specific inhibition of tyrosine phosphorylation induction by TMC triggered T cell-specific apoptosis via Bcl-2, caspase-9, caspase-3, and PARP cleavage, but not via caspase-1 activation. Protection of TMC-induced apoptosis by Bcl-2 overexpression confirmed this pathway. However, partial protection, not complete abrogation, of TMC-induced apoptosis by Bcl-2 overexpression might suggest the possible roles of other members of the Bcl-2 gene family in TMC-mediated apoptosis induction via mitochondria. Inhibition of phosphorylation of PI-3 kinase, Akt, and BAD by TMC demonstrated that Akt and BAD might be important signal communicators between TMC-mediated inhibition of T cell activation and apoptosis induction. The observation that the activated human primary T cells showed much higher sensitivity to TMC than resting T cells or activated or resting B cells is consistent with the results that TMC inhibited the tyrosine phosphorylation induction of T cell-specific signaling molecules.

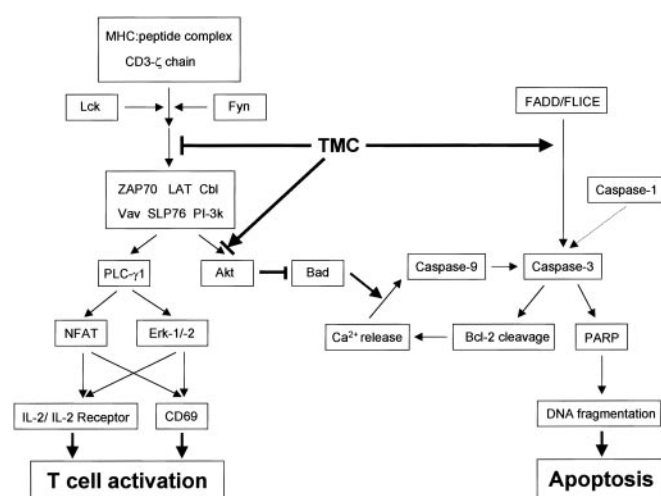
Both TMC and tautomycin exist naturally in two tautomeric forms, which resulted in similar chemical names being coined. Tautomycin, which is known to be a specific inhibitor of serine/threonine protein phosphatases PP1 and PP2A, shares a partial

structural moiety with TMC due to its tautomeric property (23). However, the functional moieties in tautomycin necessary for its inhibitory activity to PP1 and PP2A (C22-C26) and apoptosis-inducing activity (C1-C18) do not exist in TMC. Higher concentrations ( $10^3 \mu\text{g/ml}$ ) of tautomycin were required to exert a similar level of apoptosis by TMC, and T cell-specific apoptosis induction was not observed with tautomycin. In contrast to TMC, tautomycin did not show any immunosuppressive activity in *in vivo* heart allograft transplantation.

Two recent papers (24, 25) demonstrated that protein phosphatase PP1 is targeted to microtubules by the microtubule-associated protein  $\tau$ , and associates with and activates the actin-binding protein cofilin in human T lymphocytes, and regulate its polymerization through phosphorylation. Phosphorylation of cytoskeleton or its associated proteins plays an important role in T cell activation. These results strongly support the inhibitory effect of TMC on T cell activation. PP1 exists *in vivo* as multiple holoenzymes composed of catalytic subunit and several targeting or regulatory subunits, and the action of PP1 *in vivo* is thought to be controlled by the regulatory subunits. In our results, TMC might act on the unknown regulatory subunits of PP1 in T cell-specific manner.

Several possible mechanisms by which TMC induces activated T cell-specific apoptosis can be suggested. TMC might not act on the apoptotic machinery directly, but by preventing the early T cell activation pathway, it creates a form of intracellular stress and then activates mitochondria-mediated apoptosis signaling. Alternatively, inhibition of activation of an intracellular target molecule on T cell stimulation directly triggers the apoptosis signal transduction pathway via caspase-3 and -8. Thus TMC induces the cleavage of the anti-apoptotic protein Bcl-2 and the release of cytochrome *c* from mitochondria and then activation of caspase-9 complexed with Apaf-1 leading to caspase-3 activation, the executioner for apoptotic cell death. Another possibility is that inhibition of T cell activation by TMC may block the activation of Akt, the cell survival signal, leading to the activation of the pro-apoptotic molecule, BAD, by preventing its phosphorylation (Fig. 5). The *in vivo* immunosuppressive effect of TMC was tested in the heterotopic cardiac allograft rat model, and 100-fold lower concentrations of TMC than CsA increased graft survival up to more than 160 days, which is quite comparable to the *in vivo* effect of CsA. Administration of TMC up to 5 mg/kg in rats did not show any significant liver and kidney toxicity, high glucose level in biochemical analysis of blood, or histological abnormality such as renal tubular damage (Table 2).

In conclusion, we identified an immunosuppressor, TMC, from a *Streptomyces* strain from the soil of volcanic Cheju Island that acts very specifically on activated T cells. Its mechanism of action is different from that of CsA and FK506, and the level of cytotoxicity



**Fig. 5.** Proposed mechanism of action of TMC. TMC inhibits the tyrosine phosphorylation of T cell-specific intracellular signal mediator between ZAP-70 and Lck or Fyn tyrosine kinase in TcR-mediated signal transduction cascade. Inhibition of tyrosine phosphorylation by TMC induces activation of caspase-3 that can cleave the antiapoptotic protein Bcl-2 and then activation of caspase-9 complexed with Apaf leading to subsequent PARP cleavage and DNA fragmentation. Alternatively, inhibition of T cell activation may block the activation of Akt leading to activation of proapoptotic molecule, Bad.

of TMC on various organs was much lower than that of CsA when administered *in vivo*. The microemulsion preparation of TMC used in these experiments may be a suboptimal formulation, so there may be considerable room for improvement of the immunosuppressive effects. Furthermore, it can be also used in combination therapy with much lower doses of the current immunosuppressors and as a bioprobe to dissect the complex intracellular signaling pathways in T cell activation. Studies are being undertaken to identify the specific target molecules of TMC in the signal transduction pathway of T cell activation, as well as its chemical modification and optimal drug formulation for efficient clinical administration.

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